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(57) Abstract

Methods for the production of peptides, especially but not exclusively with carboxy–terminal modifications such as amidation, by recombinant means are provided.

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METHODS OF PRODUCTION OF AN AMIDATED PEPTIDE THROUGH THE USE OF A FUSION PROTEIN

The present invention is directed to the production of peptides, especially but not exclusively with carboxy-terminal modifications such as amidation, by recombinant means.

"Peptide" is a term loosely applied to a chain of amino acids, arbitrarily applied to sequences of three to over one hundred components, but possibly more, joined via their amino- and carboxy termini. There are many examples of naturally occurring peptides which function as hormones, messengers, growth factors, antimicrobials, surfactants etc and a wide variety of medicinal and other applications can be envisaged.

Currently, there are at least three major sources of peptides, extraction from natural sources, chemical synthesis and from organisms transformed with recombinant DNA constructs. The advantage of the route using transformed organisms is the biological fidelity of the synthetic process, the ability to synthesise chemically unfavourable sequences, the avoidance of chemical processes, using solvents, etc and, especially with longer peptides, cost-effectiveness.

The disadvantage of making peptides by recombinant technology is that the organisms used tend to be poor at synthesising, and if necessary secreting, short sequences of amino acids. Therefore, many of the methods considered for industrial use take advantage of fusion proteins where the short peptide sequence is made as either an amino- or a carboxy-terminal extension on another protein. Although these fusion proteins can be produced in greater quantities, and often purified by exploiting special characteristics of the fusion partner to simplify purification, difficulties can be experienced in recovering the peptide. Proteins are chemically stable molecules and therefore require specific cleavage strategies in

order to recover integral peptides with defined amino- and carboxy termini.

A wide panel of protein cleavage technologies can be envisaged. These range from chemical cleavage at specific amino acids to enzymatic cleavage using sequence-specific enzymes. Examples of chemical cleavage include cyanogen bromide cleavage after methionine residues and hydroxylamine cleavage between the amino acid pair asparagine - glycine. Examples of enzymes suitable for cutting at specific protein sequences include enterokinase, which cuts after the sequence (aspartic acid)$_4$ -lysine, and thrombin, which cuts after the basic amino acids lysine or arginine.

A common problem with both of these cleavage strategies is that sequence constraints operate on both the presence of internal sites within the peptide and the necessity to generate authentic amino-termini. For example, cyanogen bromide is only useful when there are no internal methionines in the peptide and thrombin can cut at a number of different sites after basic amino acids. Enzymatic cleavage has additional problems in terms of process economics. The enzyme must come from an acceptable and validated source (a common source of enterokinase is calf gut endothelium) and be available in economically acceptable quantity.

Carboxy-terminal amidation is a common post-translational modification found on many biologically active peptides of potential commercial interest. Examples include calcitonin, magainin and etc. . In many instances, for example, calcitonin, the natural amidated peptide is nearly two thousand times as active as the non-amidated version.

There are many different chemical and biological methods designed to produce carboxy-terminal amidated peptides. However, as with any extra process step, each

has disadvantages in terms of adding to the overall cost of the finished product.

This invention describes a method for the production of peptides as amino-terminal extensions of fusion proteins in recombinant systems. We provide novel methods whereby cleavage of the peptide from the fusion protein and modifications of the peptide such as carboxy-amidation can occur as a series of linked reactions in a single process. Such an approach benefits from the low cost and fidelity of synthesis in a biological expression system without the disadvantages posed by the necessity of a separate cleavage step.

Thus, in a first aspect, the present invention provides a method for the production of a peptide which comprises the step of expressing the peptide as part of a fusion protein followed by release of the peptide from the fusion protein by an acyl-acceptor such as a sulphur containing reductant. Suitably, at least part of the fusion protein is a molecule capable of catalysing transfer of the peptide, as an acyl moiety, to a suitable acceptor such as a proximal sulphur atom to form the thio-ester.

In preferred embodiments the peptide is chemically modified, eg amidated at its carboxy terminus after release from the fusion protein. Suitably, the amidation step is carried out in the presence of a source of ammonium ions at a suitable pH and the amidation step occurs simultaneously with release of the peptide. Examples of amidated peptides which could be prepared using these methods include Salmon Calcitonin, Human Calcitonin, Lutenising hormone releasing hormone, Oxytocin, Gastrin neuropeptide Y, Vasopressin, Corticotrophin releasing hormone, Growth hormone releasing hormone, Human Calcitonin gene related peptide, Gastrin, D-tyr-trp-gly, phe-gly-phe-gly, gly-phe-gly, Melanocyte stimulation hormone precursor, Sectetin, Thyrotrophin releasing hormone, Amylin, Substance P,

Pancreatic polypeptide, Cholecystokinin, Gastrin secretion factor, phe-his-ile, phe-tyr-tyr, Savagin, Mastoparin M, Caerulein and FMRF amide.

However, it is also possible to perform simple hydrolysis of the peptide in the absence of ammonia which would result in the formation of a free carboxylic acid terminal group. This makes the methods of the present invention suitable for the commercial production of peptides with free carboxy-termini for medicinal or other applications. Examples of such peptides include Hirulog, Magainin, thymosin alpha-1, brain naturetic peptide, atrial naturetic peptide or bactericidal/permeability-increasing protein.

The methods of the present invention can for example utilise a commercially available expression vector designed for making proteins as fusion proteins. This vector incorporates a modified self-splicing protein, an intein, making it possible to liberate the protein from its fusion partner by a simple chemical reaction. The invention utilises modified chemical conditions/steps to result in cleavage of the fusion protein thereby liberating a desired peptide, which can be modified e.g. by ambition at the carboxy-terminus.

Inteins are proteins which are expressed with flanking protein sequences at both amino- and carboy-termini. The amino- and carboxy-terminal sequences have been named exteins in keeping with the DNA nomenclature of exons and introns. A seemingly typical member of the emerging family of inteins is the VMA1 gene product from yeast. This is approximately 50kDa in molecular mass and contains essential amino acids at the amino terminal (Cysteine) and at the carboxy-terminal (histamine and asparagine). In addition, the carboxy-terminal extein must start with a cysteine. At some point after translation is completed, the amino-terminal peptide bond is broken and the extein transferred to the sulphur atom of the adjacent

cysteine to form a thio-ester. This bond is then exchanged with the cysteine at the start of the carboxy-terminal extein and then, with participation of the adjacent asparagine, exchanged with the peptide bond at this end of the intein. The overall effect of these concerted reactions is that the two exteins are seamlessly joined and the intein is released.

A detailed understanding of these reactions has emerged following the analysis of a series of mutants where essential groups at either end of the intein and the proximal ends of the exteins have been systematically replaced. This knowledge has enabled the design of mutant inteins where the amino-terminal extein can be replaced by any other protein and the self-splicing function has been disabled. However, cleavage of the resulting fusion protein is still possible by the addition of extraneous chemical agents such as the reductant dithiothreitol. The fusion protein is liberated as a thio-ester with the added reductant which gradually hydrolyses to the free acid in solution.

Calcitonin is an example of a medically and commercially important peptide suitable for manufacture using the methods described in this invention. It contains thirty-two amino acids and is amidated at the carboxy-terminus. The functional activity and amino acid sequence is highly conserved between species. Thus salmon Calcitonin , which was originally obtained mostly from natural sources but is now made by direct synthesis, is in widespread clinical use. In the past, therapies have focused on Paget's disease and hypocalcaemic shock. However, recently there has been a demand for larger amounts of material to treat osteoporosis in post-menopausal women. This application requires substantive quantities of material which makes the cost of production an increasingly important factor.

In order to make Calcitonin using the intein vector it is necessary to prepare

complimentary oligonucleotides which encode the Calcitonin sequence flanked by restriction sites designed for insertion at the appropriate site 5' to the modified intein. These sites must be chosen so that the coding sequence of the peptide is in the same coding frame as the rest of the expressed protein. Suitable oligonucleotides can be made by any number of methods, known to those skilled in the art, including most obviously direct synthesis and polymerase chain reaction amplification from a natural sequence using primers designed to contain convenient restriction sites. This DNA construct is then transformed into a suitable expression system and the resulting fusion protein harvested.

In a further refinement of the system the fusion protein also comprises a label, which allows for identification and/or purification of the fusion protein, and thus the peptide, by affinity or other chromatographic methods. Examples of a suitable label include a specific chitin-binding domain, or part thereof, a repeat of acidic or basic amino acids, a poly-histidine sequence, glutathione S transferase and lysozyme. For example, the carboxy-terminus of an intein can be fused with a specific chitin-binding domain. This binds tightly to a packed column of chitin beads and can be used for the affinity-purification of the intact fusion protein. After extensive washing, the column can then be treated with an appropriate cleavage reagent and the liberated target peptide eluted.

Any expression system which can operate on a commercial scale is suitable although the intein based vector described above is designed for use in E. coli. Other vectors can be designed for optimal use in a particular expression system. For example, if a mammalian expression system was chosen, then protein-encoding regions should have optimised codon usage for that particular system. Expression could also be improved by use of a smaller affinity tag for identification and/or purification such as a repeat of acidic or basic amino acids as described above, to

permit resolution from contaminating proteins by ion-exchange chromatography or by the inclusion of a poly-histidine sequence for purification on a metal chelate matrix. A further modification which could improve secretion from a mammalian system (the current E. coli vector is designed for intracellular protein production) would be to add a secretory leader sequence to the calcitonin to promote secretion into the media or into the milk of transgenic animals. Suitably, such a leader sequence should be removed during the secretory process by natural processing enzymes.

Examples of expression systems which could be used to express peptide fusion proteins include bacteria (*E.coli*, *B.subtilis* etc.), yeast (*S. cerevisiae*, *P.pastoralis* etc.), insect cells (*S. frugiperda*), mammalian expression systems (chinese hamster ovary, baby hamster kidney etc.), transgenic mammalian expression in milk or other body fluids (preferably pig, cow, sheep, goat, rabbit etc) and plants (potato, corn, etc). In the case of an *E.coli* expression system, the initiator methionine will be retained in the expression product. Thus, where the peptide of interest is one which does not include an additional methionine in its sequence, this initiator methionine can be removed using cyanogen bromide. One example of such a peptide is Calcitonin.

Expression could be optimised for any of these systems, and for intracellular or extracellular production, by the appropriate selection of leader sequence, codon usage, intein or mutant thereof, and purification strategy. The skilled person will appreciate that this invention is not tied to any particular manifestation of intein or any species as a source. For instance, it may not be necessary to use a whole intein molecule, much of the sequence may be irrelevant to the desired process and perhaps most of the molecule is functionally unnecessary. Indeed, other proteins outside the definition of "intein" may be capable of transferring the peptide bond at

the carboxy-terminus of the target peptide to an appropriate thiol group thus creating the thio-ester group which is necessary for cleavage with concomitant amidation.

Thio-esters are relatively reactive chemical groups, compared to either peptide bonds or oxygen-esters, and are therefore readily converted to amides under mild reactive conditions. There are two points in the normal cleavage and release pathway during which the fused peptide can be converted to a carboxy-terminal amide. The first and probably most suitable point is after the peptide has been released from the fusion partner by the addition of a thiol reagent. The preferred reagent is dithiothreitol but any number of sulphur-containing reductants could also function effectively. This reaction is essentially a thiol-interchange reaction where the thiol-ester formed between the carboxy-terminus of the peptide and the sulphur of the intein cysteine is transferred to one of the dithiothreitol sulphur atoms. As with any chemical reaction, the acyl shift reaction, between the amine of the cysteine at the amino-terminus of the intein and the sulphur of the same amino acid residue, is an equilibrium. With the yeast intein described above this equilibrium is shifted in favour of the amine group and the thio-ester is a minor component.

The added thiol reagent removes this thio-ester species and therefore drives the reaction in the direction of making more thio-ester until effectively all of the peptide is released as free thio-ester. The released thio-ester is relatively stable to hydrolysis by water (which would generate the unwanted free acid) and is thus suitable for cleavage by any chemical conditions which will promote amide formation.

The second point where the peptide exhibits a thio-ester is to the intein itself but as described above, this species is a minor component. However, even here it would

be possible to design chemical conditions to allow simultaneous release of the peptide as an amidated species.

The conditions expected to favour thio-ester cleavage with concomitant amidation are many and the following is only meant to illustrate a representitive selection of the possible reagents and reactions. Chemically, amides can be formed by the cleavage of thio-esters with ammonia and related compounds. This requires conditions where the positive charge of the carbonyl is enhanced (which is an effect of the adjacent sulphur atom) and the lone pair of electrons on the nitrogen of ammonia are available. In aqueous solution the positively-charged ammonium ion, provided by a salt such as ammonium phosphate or sulphate, is in equilibrium with uncharged ammonia, the reactive species, and the concentration of free ammonia is thus increased with a lowering of the hydrogen ion concentration. It is therefore expected that the reaction promoting the formation of the amide product, although likely to proceed at relatively low pH values, for example pH 4.0 to 6.0, will occur more rapidly as the pH is increased in the range 6.0 to 9.0 or even 10.0, where the equilibrium is shifted significantly in favour of ammonia formation.

The optimal range will be a compromise between the highest pH which will be tolerated by the peptide substrate itself and the lowest pH whereby the reaction still proceeds at an acceptable rate. This optimum range will be determined by the sequence of the peptide itself and other factors relating to the properties of the fusion partner and to process-related, especially purification, issues. Similar conditions and constraints are likely to apply whether the cleavage/amidation reactions occur simultaneously or sequentially. There are many other chemical conditions which can be envisaged by those skilled in the art, both in aqueous and non-aqueous systems, which could achieve the desired reactions. The above is meant simply as an illustration of a suitable method and is not intended to exclude

these other possible approaches.

The invention will now be described by way of the following examples, which should not be construed as limiting the invention in any way.

Example 1 : Glycine Extended Salmon Calcitonin

1.1. Cloning Strategy

Vector pCYB1, obtainable from New England Biolabs, containing a NdeI site for translation initiation and a SapI site directly adjacent to the intein, was used to clone and express glycine extended salmon calcitonin (sCT-G). The sCT-G coding sequence was synthesised as two complementary single stranded oligonucleotides of 103bases and 104bases. The codon usage was optimised for expression in E.coli. Annealing of the two strands produced 5' overhangs complementary to the NdeI (5' end) and the SapI site (3' end). The double stranded oligonucleotide was inserted into pCYB1 digested with NdeI and SapI. The expression of the fusion gene is under the control of the P_{tac} promoter and is regulated by IPTG due to the presence of a $lacI^q$ gene on the vector.

1.2. Fusion Protein Expression and Analysis

The pCYB1 vector containing sCT-G was transfected into DH5-α, cells grown, induced with IPTG, harvested and lysed by sonication. Expressed fusion was captured on chitin agarose which was washed and then boiled in SDS-PAGE sample buffer. The supernatant was run on 16% SDS-PAGE gels and the protein visualised with coomassie stain or electroblotted to PVDF membrane for N-terminal sequencing. The sequence analysis indicated that the sCT-G was N-terminally truncated at two positions; Ser2 and Thr6.

1.3. Fusion Protein Cleavage and Peptide Amidation

Chitin agarose bound fusion was washed with 20mM Hepes pH 8.0, 40mM DTT (cleavage buffer A) or with cleavage buffer A supplemented with 3.0M ammonium bicarbonate (cleavage buffer B) and incubated at 4°C overnight. Released sCT-G was washed from the column and captured on a cation exchange resin then eluted with a salt step. C18 RP-HPLC analysis after digestion with trypsin showed that the product formed with cleavage buffer B contained greater than 90% amidated C-terminus while the product with cleavage buffer A had a mixture of carboxylic C-terminus and an adduct extended by a single Cys residue, presumably from the intein N-terminus (Figure 1).

Example 2 : Leutenizing Hormone Releasing Hormone (LHRH)

2.1 Cloning Strategy

Cloning was preformed exactly as described for sCT-G except that the oligonucleotides contained the LHRH coding sequence (Tan, L. and Rousseau, P. *Biochem. Biophys. Res. Com.* **109**: 1061-1071 (1982)).

2.2. Fusion Protein Expression and Analysis

As described for sCT-G. N-terminal sequencing demonstratred the LHRH was extended at the N-terminus by a single Met residue, retained from the E.coli initiation signal.

2.3. Fusion Protein Cleavage and Peptide Amidation

The LHRH fusion was treated in the same manner as the sCT-G fusion until the final cation capture step. The column wash was applied directly to an elecrospray

mass spectrometer and the data reconstructed to give the mass of the parent ion (Figure 2). LHRH from cleavage buffer B (as described in example 1) resulted in a parent ion with a mass of 1331Da consitent with the Met extended, amidated molecule. LHRH from cleavage buffer A (as described in example 1) gave a parent ion mass of 1332 Da consistent with the Met extended free acid. The difference of 1Da is the expected mass difference between an amide and carboxylic acid.

EXAMPLE 3

Cloning of Human Amylin

The *IMPACT I* (Intein Mediated Purification with an Affinity Chitin-binding Tag) protein purification system from New England Biolabs (NEB) offers 4 *E. coli* expression vectors, which differ in their available cloning sites. Human Amylin is cloned using the NEB vector pCYB1, which contains a *Nde*I site for translation initiation and a *Sap*I site directly adjacent to the intein.

The Human Amylin sequence is synthesised as two complementary single stranded oligo nucleotides of 115 and 116 bases respectively. The codon usage is optimised for expression in *E. coli*. Annealing of the two strands produces 5' overhangs complementary to the *Nde*I (5' end) and the *Sap*I site (3' end). The double stranded oligo nucleotide can be inserted directly into pCYB1 which has previously been digested with both *Nde*I and *Sap*I.

PCYB1:

5' CAT ATG GCT AGC.....GGC TCT TCC TGC TTT 3'

3' GTA TAC CGA TCG.....CCG AGA AGG AGC AAA 5'
NdeI *SapI*

Human Amylin:

NdeI BspMI
CATATGAAATGCAACACCGCGACCTGCGCGACCCAGCGCCTGGCG
GTATACTTTACGTTGTGGCGCTGGACGCGCTGGGTCGCGGACCGC

MboI *DdeI*
AACTTCCTGGTGCATAGCAGCAACAACTTCGGCGCGATCCTGAGC
TTGAAGGACCACGTATCGTCGTTGTTGAAGCCGCGCTAGGACTCG

AGCACCAACTGGGCAGCAACACCTATTGCTTT
TCGTGGTTGACCCGTCGTTGTGGATAACGAAA

EXAMPLE 4

General Protcol for expressing peptide fusion proteins in *E.coli* and purification of fusion protein and released peptide

Expression in bacteria requires transformation of cells with an expression construct using any one of a range of standard methods (Maniatis *et al*, *supra*). After cell growth, it is usual to induce expression of the target fusion protein using a combination of an inducible promoter, for example the β-galactosidase promoter, and a small molecule inducer such as IPTG. The fusion protein is then recovered after cell harvesting and breakage and then purified by affinity

chromatography. Most usually, this involves passing the clarified cell lysate through a column of an appropriate affinity matrix displaying a ligand to which the fusion protein binds. Contaminants are then washed from the matrix before either specific elution of the fusion protein or cleavage of the bound fusion protein *in situ*. For instance, with the Impact vector described in Examples 2 and 3, the fusion protein containing lysozyme would be purified by cation exchange chromatography. In this case, cleavage *in situ* is probably not an option, unless cleavage conditions can be found which do not promote elution of the fusion protein. Under these circumstances, cleavage in solution phase would be required. Cleavage of the fusion protein whilst bound to a matrix simplifies the subsequent purification of the peptide.

Cleavage of the fusion protein can be done by the direct addition of a thiol acyl-acceptor, such as 10mM DTT, to yield a thioester intermediate, which can subsequently be converted to the amide by treatment with ammonia salts at a pH above 6.0. Simultaneous cleavage and conversion t an amide may also be possible with the addition of a suitable mixture of acceptor thiol and ammonia salt.

Released peptide is then further purified, if necessary, using conventional techniques such as solvent partitioning and HPLC.

CLAIMS:

1. A method for the production of a peptide, which comprises the step of expressing the peptide as part of a fusion protein, followed by release of the peptide from the fusion protein by an acyl-acceptor, such as a sulphur containing reductant.

2. A method as claimed in claim 1 wherein at least part of the fusion protein is a molecule capable of catalysing transfer of the peptide, as an acyl moiety to a suitable acceptor, such as a proximal sulphur atom to form a thio-ester.

3. A method as claimed in claim 1 or claim 2 wherein the peptide is amidated at its carboxy terminus after release from the fusion protein.

4. A method as claimed in claim 3 wherein the amidation step is carried out in the presence of a source of ammonium ions at a suitable pH.

5. A method as claimed in claim 3 or claim 4 wherein the amidation step occurs simultaneously with release of the peptide

6. A method as claimed in any one of claims 1 to 5 wherein the peptide is Salmon Calcitonin, Human Calcitonin, Lutenising hormone releasing hormone, Oxytocin, Gastrin neuropeptide Y, Vasopressin, Corticotrophin releasing hormone, Growth hormone releasing hormone, Human Calcitonin gene related peptide, Gastrin, D-tyr-trp-gly, phe-gly-phe-gly, gly-phe-gly, Melanocyte stimulation hormone precursor, Sectetin, Thyrotrophin releasing hormone,, Amylin, Substance P, Pancreatic polypeptide, Cholecystokinin, Gastrin secretion factor, phe-his-ile, phe-tyr-tyr, Savagin, Mastoparin M, Caerulein or FMRF amide.

7. A method as claimed in claim 6 wherein the peptide is Salmon Calcitonin or Human calcitonin.

8. A method as claimed in any one of claims 2 to 7 wherein the fusion protein comprises at least part of a modified intein sequence.

9. A method as claimed in claim 8 wherein the modification of the intein sequence, or part thereof, results in disablement of the self-splicing function.

10. A method as claimed in claim 8 or claim 9 wherein the intein sequence, or part thereof, is derived from the VMA1 gene from yeast.

11. A method as claimed in claim 1 or claim 2 wherein the peptide is released from the fusion protein by hydrolysis.

12. A method as claimed in claim 11 wherein the peptide is Hirulog, Magainin, thymosin alpha-1, brain naturetic peptide, atrial naturetic peptide or bactericidal/permeability-increasing protein.

13. A method as claimed in any one of claims 1 to 12 wherein the fusion protein comprises a label, which allows for identification and/or purification of the fusion protein by affinity or other chromatographic methods.

14. A method as claimed in claim 13 wherein the label is an affinity label.

15. A method as claimed in claim 14 wherein the affinity label comprises a specific chitin-binding domain, or part thereof, a repeat of acidic or basic amino acids, a poly-histidine sequence, glutathione synthetase and lysozyme.

16. A method as claimed in any one of claims 1 to 15 wherein the fusion protein is expressed in bacteria, yeast, plant tissue, including whole plants, insect cells, mammalian cells or in a body fluid of a transgenic mammal.

17. A method as claimed in claim 15 wherein the fusion protein is expressed in *E.coli* or *B.subtilis*.

18. A method as claimed in claim 17 wherein the fusion protein is expressed in *E.coli* and wherein the peptide is treated with cyanogen bromide if it does not contain a methionine in its sequence.

19. A method as claimed in claim 15 wherein the fusion protein is expressed in *S.cerevisiae* or *P.pastoralis*.

20. A method as claimed in claim 15 wherein the fusion protein is expressed in chinese hamster ovary cells or baby hamster kidney cells.

21. A method as claimed in claim 15 wherein the fusion protein is expressed in transgenic potato tissue or transgenic corn tissue.

22. A method as claimed in claim 15 wherein the fusion protein is expressed in the milk of a transgenic pig, cow, sheep, goat or rabbit.

23. A method as claimed in claim 15 wherein the fusion protein is expressed in insect cells, e.g. in the *S. frugiperda* cells.

24. A method as claimed in any one of claims 1 to 23 wherein the sequence

coding for the fusion protein also includes a secretory leader sequence.

25. A method as claimed in claim 24 wherein the secretory leader is removed by natural processing enzymes during secretion.

26. A DNA construct coding for a fusion protein as defined in any one of claims 1, 2, 6 to 15, 24 or 25.

27. A DNA construct as claimed in claim 26 which is in the form of a vector.

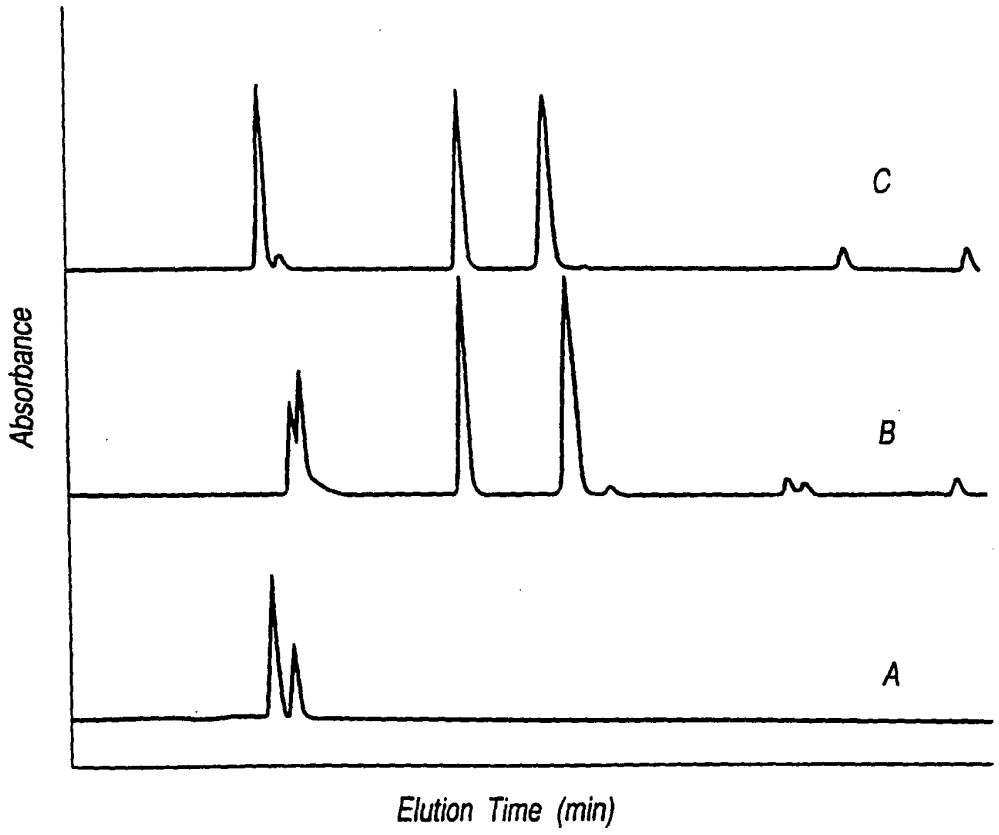
28. A host cell transformed or transfected with a DNA construct as defined in claim 26 or claim 27.

29. A host cell as claimed in claim 28 modified by any one or more of the features of claims 16 to 21.

30. A transgenic, non-human, mammal, which has incorporated in its genome a DNA construct as, defined in claim 26.

31. A transgenic mammal as claimed in claim 29 which is a transgenic pig, cow, sheep, goat or rabbit.

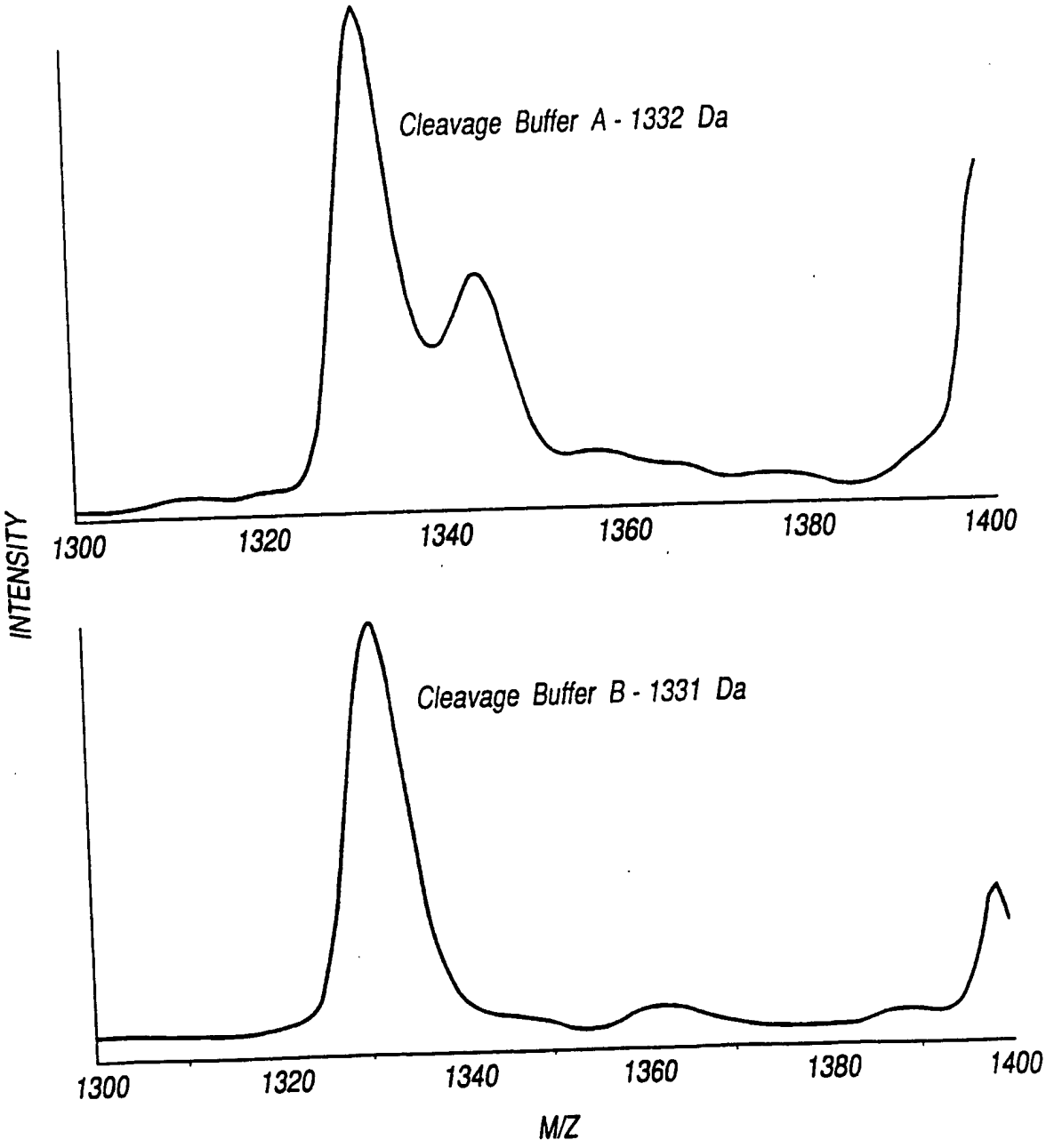
C18 RP - HPLC Analysis of sCT - G after Digestion with Trypsin



SCT - G from DTT only (B) and DTT + Ammbic (C) were analyzed by C18 RP-HPLC following digestion with trypsin. The C - terminal peptide retention times (eluting first) were compared with those synthetic amidated (A peak 1) and free acid (A peak 2) standards.

Fig.1

Electrospray Mass Spectroscopy Analysis of LHRH Products



Cleavage Buffer A - 20mM Na Hepes pH8.0, 40mM DTT
Cleavage Buffer B - 20mM Na Hepes pH8.0, 40mM DTT, 3M Ammbic

Fig.2

INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/GB 98/01281

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K19/00 C07K1/00 C07K7/23 C07K14/575
A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHONG S ET AL: "Protein splicing involving the Saccharomyces cerevisiae VMA intein. The steps in the splicing pathway, side reactions leading to protein cleavage, and establishment of an in vitro splicing system." J BIOL CHEM, SEP 6 1996, 271 (36) P22159-68, XP002076973 UNITED STATES	1-3, 8-10,16, 17
Y	see abstract; figure 1 see page 2160, right-hand column, paragraph 3 - paragraph 4	4-7,11, 12,18-31

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
10 September 1998	22/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/GB 98/01281

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 01642 A (NEW ENGLAND BIOLABS INC ;COMB DONALD G (US); PERLER FRANCINE B (US) 16 January 1997 see page 13, paragraph 2; claims 1-49; examples 9,15	1-3, 8-10, 13-17
Y	WO 94 01451 A (BIONEBRASKA INC) 20 January 1994 see claim 22; table 1	4,5,18
Y	WO 95 27782 A (PPL THERAPEUTICS SCOTLAND LTD ;COTTINGHAM IAN ROBERT (GB); GARNER) 19 October 1995 see claims 1-26	6,7,20, 22-31
Y	EP 0 552 559 A (UNILEVER PLC ;UNILEVER NV (NL)) 28 July 1993 see claims 1-10	11,12, 19,21
P,X	CHONG S ET AL: "Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element." GENE, JUN 19 1997, 192 (2) P271-81, XP002076974 NETHERLANDS see the whole document	1-3

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. onal Application No
PCT/GB 98/01281

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9701642 A	16-01-1997	EP 0835323 A	15-04-1998
WO 9401451 A	20-01-1994	AU 673190 B	31-10-1996
		AU 4772593 A	31-01-1994
		CA 2140002 A	20-01-1994
		EP 0651761 A	10-05-1995
		JP 7509128 T	12-10-1995
		NZ 255041 A	26-11-1996
		US 5635371 A	03-06-1997
		US 5656456 A	12-08-1997
WO 9527782 A	19-10-1995	AU 2112095 A	30-10-1995
		CA 2187173 A	19-10-1995
		EP 0753059 A	15-01-1997
		JP 10502243 T	03-03-1998
EP 0552559 A	28-07-1993	NONE	